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XAP2 inhibits glucocorticoid receptor activity in mammalian cells

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ABSTRACT

XAP2 is member of a protein family sharing the TPR protein interaction motif. It displays close homology to the immunophilins FKBP51 and FKBP52 that act via the Hsp90 folding machinery to regulate the glucocorticoid receptor (GR). We show that XAP2 inhibits GR by reducing its responsiveness to hormone in transcriptional activation. The effect of XAP2 on GR requires its interaction with Hsp90 through the TPR motif. The PPIase-like region turned out to be enzymatically inactive. Thus, PPIase activity is not essential for the action of XAP2 on GR, similarly to FKBP51 and FKBP52.

1. Introduction

Corticosteroids show a broad variety of physiological functions [1]. Their effects are mainly mediated via two members of the nuclear receptor family [2], the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). It is generally accepted that GR resides predominantly in the cytoplasm in the absence of hormone as part of a large chaperone heterocomplex which contains amongst others an Hsp90 dimer, p23 and one member of the TPR-(tetratricopeptide repeat) protein family such as FKBP51 or FKBP52 [3,4]. Upon ligand binding, GR dissociates from the heterocomplex and translocates into the nucleus where it regulates transcription either by binding to its cognate DNA elements or by interacting with other transcription factors [5].

Chaperones regulate GR function at least at three distinct levels. The folding of GR in the cytoplasm by molecular chaperones has been intensely investigated [4,6], but evidence has also been provided that they are involved in nuclear translocation of GR [7–9] and in disassembly of its transcriptional complexes [10]. The highly homologues immunophilins FKBP51 and FKBP52 display differential effects on GR folding and nuclear translocation [7,11]. FKBP51 has recently attracted particular interest, because it was found to be genetically associated with various stress-related disorders and phenotypes [12–17]. This is intriguing, because the role of GR in a range of stress-related diseases is well documented [18].

The hepatitis virus B X-associated protein 2 (XAP2) is a 37 kDa-protein with wide distribution in human tissues that was initially identified as an interacting partner of the hepatitis virus B X-protein [19]. It is also known as AhR (arylhydrocarbon receptor)-interacting protein (AIP), AhR-associated protein 9 (ARA9) or FKBP23. XAP2 contains three TPR-domains and a PPIase-like domain in the N-terminus and shows homology to FKBP51 and FKBP52 [20,21]. In contrast to these immunophilins, XAP2 does not bind to FK506 [11]. Nevertheless, the enzymatic activity of its PPIase-like domain is considered unclear at present [22]. XAP2 has been described to act on AhR in concert with Hsp90 in yeast as well as in mammalian cells [20,23–26]. XAP2 was reported to reduce ubiquitination of the receptor and to regulate its subcellular distribution, albeit there is some controversy about the exact details [27–29]. More recently, it has been shown that XAP2 interacts with and is required for, another member of the family of the β activation of thyroid hormone receptor 1 nuclear receptors [30].

Because of its homology to established GR regulators, we tested potential effects of XAP2 on GR in mammalian cells. We show that XAP2 inhibits GR transcriptional activity and influences intracellular distribution of the receptor in a time-dependent manner. Moreover, we show that the PPIase-like domain of XAP2 displays no enzymatic activity. This is in line with the effects of FKBP51 and FKBP52 on GR, which are independent of their PPIase activity.

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2. Materials and methods

2.1. Cell culture, transfection, and reporter gene assays

Cultivation and transfection of human neuroblastoma SK-N-MC cells, HEK cells and HeLa cells were as described previously [7]. Two days before transfection, cells were seeded in steroid-free medium.

Transfection was by ExGen (Fermentas) according to the manufacturer's instructions. Unless indicated otherwise, about 50,000 cells were transfected with 40 ng MTV-Luc-, 5–30 ng pCMV-Gaussia-luciferase and 25 ng PRK7-GR-plasmid. The overall amount of transfected plasmids was equaled for each condition by supplementing "empty" expression vector. After transfection (0–8 h), cells were seeded in steroid-free medium containing either hormone or the respective solvent for 16 h.

Firefly luciferase and Gaussia luciferase assays were as described [31] and as recommended by the manufacturer (PJK GmbH).

2.2. Plasmids

MTVLuc-, FLAG-GR, GFP-GR-, FKBP51 WT- and FKBP51 TPR-MUT-plasmids have been described previously [7].

XAP2 WT-plasmids were a kind gift of I. Pongratz (Karolinska Institute, Stockholm, Sweden). The TPR-mutation in FLAG-XAP2 (K266A, R271A) within the TPR-domain by alanines was introduced by using the QuickChange Site-Directed Mutagenis Kit (Stratagene).

2.3. Immunoprecipitation and Western blot

HEK cells were transfected by electroporation with 10 μg of a plasmid expressing a FLAG-tagged form of XAP2WT, XAP2TPRMUT, or FKBP51WT, or the empty expression vector in the control condition. For the competition experiment (Fig. 2C) 1–2 μg of XAP2FLAG plasmid were transfected along with increasing amounts of FKBP52, or cloning vector to keep the overall amount of plasmid constant. Cell lysis, immunoprecipitation and immunoblots were as described previously [7].

2.4. Fluorescence analysis

HeLa cell cultivation, transfection with GFP-GR together with either XAP2WT or control vector, and evaluation of the results was as described previously [7]. Between 167 and 193 cells were evaluated for each experimental condition.

2.4.1. XAP2 expression and purification

The PPIase-like domain of Xap2 comprising amino acids 1–121 (XAPfrag121) was cloned into the pMAL cRI vector (New England Biolabs) to create a soluble XAPfrag121-MBP fusion protein. This protein was then expressed in *Escherichia* coli (pBL3Lys, Invitrogen) cultivated in LB media with 0.3 mM isopropyl-ß-D-C-thiogalactopyranoside (IPTG) for 2 h at 30 °C. Purification was performed over an amylose resin column (New England Biolabs) as described [32]. In some fractions MBP was cleaved off by Factor Xa (New England Biolabs). Protein concentrations were determined with the Bradford assay (BCA kit, Pierce) and estimated in parallel by comparison with a marker protein in a Coomassie gel. As positive control, FKBP52 and FKBP12 were cloned into a pProExHTa vector, expressed in *E. coli* and purified on a Ni-NTA agarose column according to the manufacturer's recommendations (Qiagen).

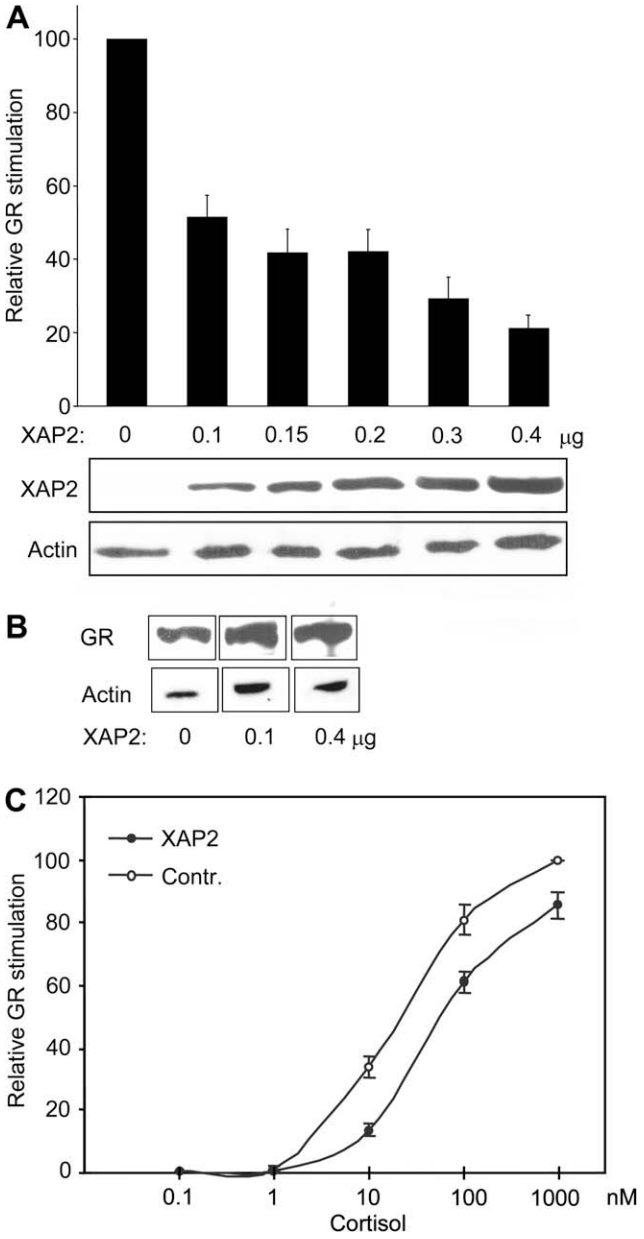


Fig. 1. XAP2 inhibits the transcriptional activity of GR. (A) SK-N-MC cells were transfected with the reporter plasmid MTV-Luc, the Gaussia luciferase control plasmid, a GR expressing plasmid and the XAP2 expression plasmids at increasing amounts. Cells were cultivated over night in the presence of 10 nM cortisol or the solvent ethanol and then harvested for determination of firefly luciferase and Gaussia luciferase activities. Data are presented as relative stimulation ±S.E. of 7 independent experiments. Lower panel: Western control of XAP2 expression in representative lysates used in A. (B) Western blot control of GR expression in representative lysates used in A. (C) The inhibitory action of XAP2 on GR depends on the concentration of cortisol.

2.4.1.1. Active site titration. The binding of XAPfrag121 to the immunophilin ligand rapamycin was analysed in a fluorescence polarization assay [33]. Fluorescent ligand (Rap-Fl, Kozany et al., [43]) was used in three different concentrations (3, 12 and 48 nM) in assay buffer (20 mM HEPES, 0.01% Triton X-100, pH 8.0) to measure titration curves for FKBP52 as a positive control. A concentration of 48 nM of the fluorescent ligand was used for the titration curve with XAPfrag121 ($n = 2$). After incubation at room temperature for 30 min the fluorescence polarization was measured in a Tecan reader (Genios Pro). The binding curves were analysed using Sigma Plot9. For the analysis of K_d values, the data

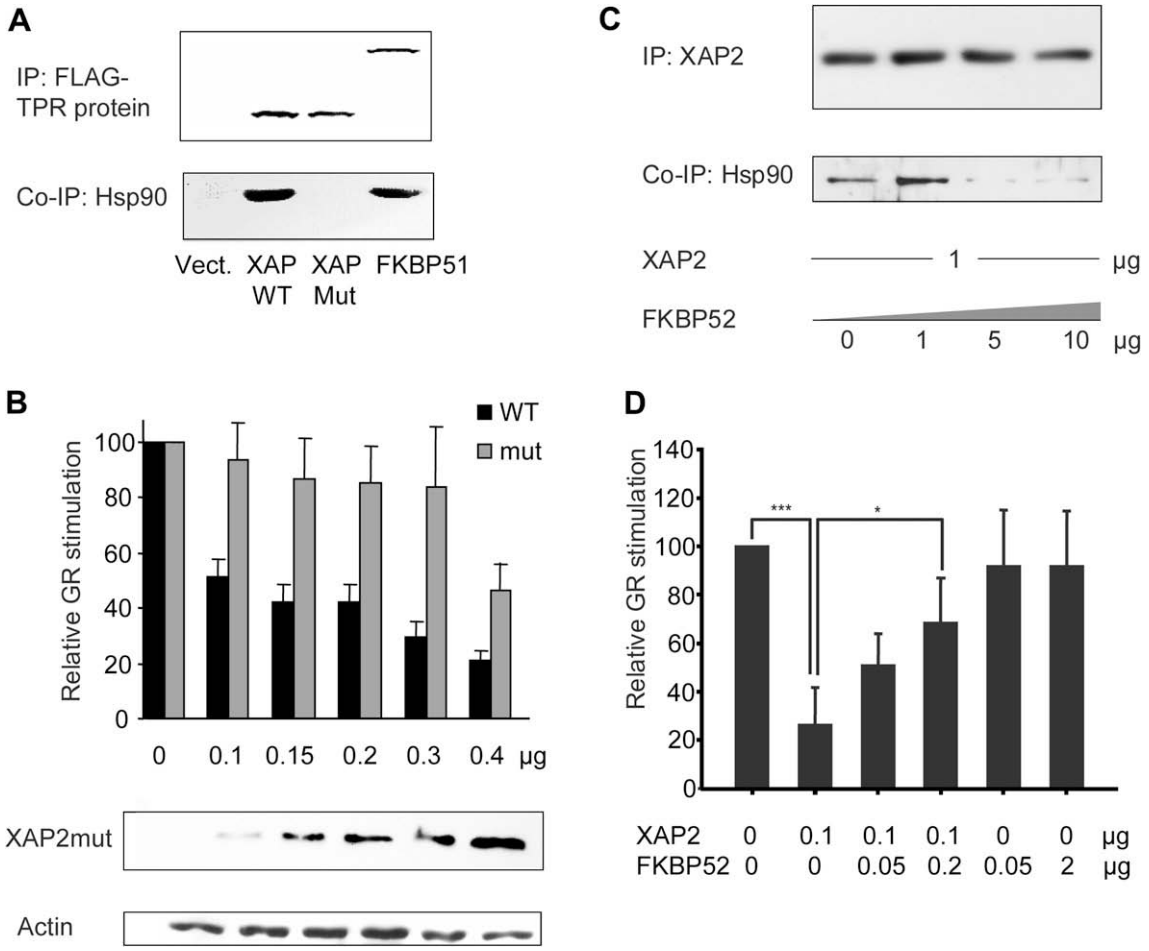


Fig. 2. The activity of XAP2 on GR is Hsp90-dependent. (A) Plasmids coding for a FLAG-tagged form of XAP2WT, XAP2MUT (K266A, R271A), and FKBP51 or empty vector as controls were transfected into HEK cells. Interaction with Hsp90 was probed in lysates obtained the next day. (B) Reporter gene assay as in Fig. 1A to test the influence of the TPR-mutated XAP2 on GR. Lower panel, expression control. (C) Co-immunprecipitation like in (A) to probe the Hsp90 interaction of XAP2 in the absence or presence of increasing amounts of FKBP52 as competitor. (D) Reporter gene assay as in Fig. 1A to test the ability of FKBP52 to counteract the inhibitory action of XAP2 on GR. Amounts of (co-)transfected plasmids are indicated. $^{*}p > 0.05$, $^{***}p < 0.001$.

were fitted to the following equation [34,43]: $A = [(A_{max} - A_{min})/[L]_t]\{[([R]_t + [L]_t + K_d)/2] - \sqrt{\{[([R]_t + [L]_t + K_d)/2]^2 - [L]_t[R]_t\}}\} + A_{min}$. ($A_{max}$: maximal anisotropy value, A_{min}: minimal anisotropy value, K_d: dissociation constant of the used ligand, $[L]_t$: total concentration of used ligand, $[R]_t$: total concentration of titrated protein receptor.)

2.4.2. PPIase assay

The PPIase activity was assessed by a spectrophotometric chymotrypsin-coupled enzymatic assay as described [35]. In this assay the peptide substrate Suc-ALPF-pNitroanilide is reversibly cis–trans isomerised by a PPIase. In a second step the trans-isomer is hydrolysed by chymotrypsin, which results in release of pNitroanilide and a consecutive increase in absorbance at 390 nM. The C in a buffer containing 50 mM HEPES and 100 mM NaCl assay was conducted at 4 (pH 8.0). The peptide substrate was dissolved at 4 mM in a solution of 470 mM LiCl in dry trifluoroethanol and stored under argon to exclude water vapor. Substrate was added at a final concentration of 100 µM immediately before measurements in a spectrophotometer (Beckmann DU530). Absorbance was then monitored until the reaction was complete (2–10 min). Curves were analysed using Sigma Plot9 and fitted with a three parameter fit for an exponential rise to a maximum ($y = y_0 + a(1-e^{-bx})$).

To avoid interference by a potential chymotrypsin sensitivity of the proteins, the order of addition of the assay components was changed. The peptide substrate in dry trifluoroethanol/470 mM LiCl was added to XAP2, FKBP52 or no protein control in the absence of chymotrypsin at the concentrations described above. After 3 min incubation at 4 °C, chymotrypsin was added and the progress curves were fitted as above to determine the trans configuration at the start of the chymotrypsin reaction (pNA burst).

3. Results

3.1. XAP2 inhibits GR-dependent transcription in mammalian cells

To investigate the effect of XAP2 on the transcriptional activity of GR, we used a GR-responsive reporter assay [7]. In two different cell lines, the neuroblastoma cell line SK-N-MC and HEK cells, we observed impaired GR-mediated transcription the more XAP2 was ectopically expressed (Fig. 1A). As over-expression of XAP2 did not lead to a decrease of GR-protein levels in our test system (Fig. 1B), we could exclude receptor degradation as a possible mechanism of the observed XAP2-dependent inhibitory effect on GR.

Moreover, XAP2 inhibited GR less efficiently at higher concentrations of hormone (Fig. 1C). This suggests that the inhibitory effect of XAP2 on GR activity is at least partially due to a reduction in ligand-binding affinity of the receptor.

3.2. The inhibitory effect of XAP2 requires interaction with Hsp90

Hsp90-dependence has been demonstrated for the FKBP51-induced inhibition of GR [3]. For the interaction of XAP2 with Hsp90, the integrity of the TPR domain is essential [8,23,24]. To create an XAP2-protein that lacks Hsp90-interaction, we replaced the positively charged amino acids lysine (266) and arginine (271) within the TPR-domain by alanines, following a similar strategy that successfully disabled Hsp90 interaction of other TPR proteins such as FKBP5 and PP5 [7,36,37]. We verified that the TPR-mutated XAP2 lost the ability to interact with Hsp90 by co-immunoprecipitation (Fig. 2A). This mutant did also not inhibit GR-dependent transcriptional activity at protein levels that, in the case of wtXAP2, were sufficient to reduce GR activity (Fig. 2B). At the highest amount of expression plasmid used, some effect on GR was observed, which may be due to residual Hsp90 binding of the mutated XAP2. A similar result was observed with a plasmid encoding for TPR-mutated FKBP51 (data not shown).

To show the competition between other Hsp90 binding TPR-cofactors and XAP2 more directly, we expressed XAP2FLAG together with increasing amounts of FKBP52 in HEK cells. Co-precipitation of XAP2 shows comparable amounts of XAP2 precipitation throughout the different conditions. Increasing amounts of co-expressed FKBP52 resulted in decreasing amounts of Hsp90 co-precipitated with XAP2 (Fig. 2C).

To test the consequences of this competition on the transcriptional activity of GR, we co-expressed XAP2 together with increasing amounts of FKBP52 in a reporter gene assay. We observed that FKBP52 was indeed able to rescue GR activity, which had been diminished by XAP2 (Fig. 2D). Co-expression of FKBP52 in the absence of XAP2 did not influence GR activity, as described previously [7].

Thus, XAP2 influences GR via the Hsp90 heterocomplex.

3.3. XAP2 delays nuclear accumulation of hormone-bound GR

An inhibitory influence of XAP2 on the subcellular distribution of AhR in the presence as well as in the absence of ligand has been reported [27,29]. Moreover, we have found that FKBP51 significantly reduces nuclear translocation of GR and disrupts interaction of the GR heterocomplex with the motor protein dynein [7].

To investigate a possible effect of XAP2 on nuclear accumulation of GR, we followed the subcellular localisation of a GFP-tagged GR-protein with or without co-transfected XAP2 in HeLa cells after incubation with 10 nM cortisol for up to 60 min. The distribution of GR between cytosol and nucleus was monitored under a fluorescent microscope. For each experimental condition, >100 cells were classified by a blinded and independent scientist. Cells were scored from 1 (exclusively cytoplasmic detection) to 5 (exclusively nuclear detection) as described in the literature [27]. In the absence of ectopic XAP2, we observed the expected hormone dependent nuclear accumulation of the receptor (Fig. 3). When co-expressing XAP2, we detected no significant difference in GR-subcellular localisation in the absence of ligand (Fig. 3). After addition of hormone, we observed a time-dependent reduction of the nuclear accumulation of GR in cells with XAP2 with a maximum at 20–40 min. After 60 min, XAP2 had no influence on the subcellular localisation of GR. Thus, GR accumulation in the nucleus is not blocked, but rather delayed.

3.4. The PPIase-like domain of XAP2 does not bind to rapamycin and shows no PPIase activity

So far, it has been unclear whether the PPIase-like domain of XAP2 is enzymatically active [22,23]. There are several immunophilin ligands known, which bind to the PPIase domain of the immunophilins and inhibit PPIase activity. Amongst the most commonly used ones are FK506 and rapamycin, and FK506 has already been found not to bind Xap2 [23]. Using a fluorescence polarization assay, we show here that rapamycin does not bind to Xap2 either (Fig. 4A). FKBP52 was used as a positive control for the efficient binding of the fluorescent dye-tagged rapamycin (Fig. 4A). At a concentration of 3 nM Rap-Fl, the approximate K_d of rapamycin binding to FKBP52 was calculated as 2.8 nM, which is in the range of previously published data ([38]: K_d = 6.12 nM; [39]: K_d = 1.4 nM).

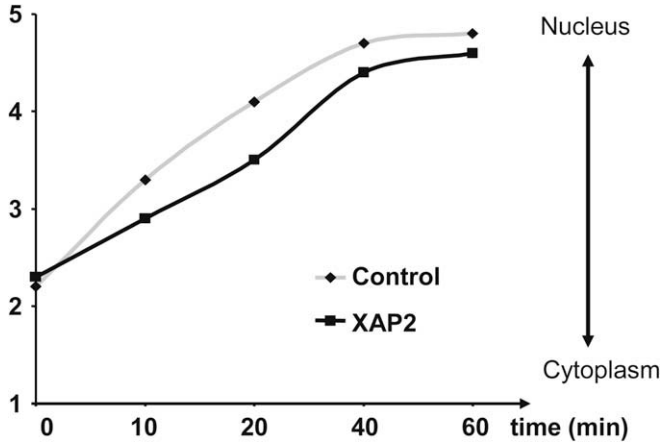


Fig. 3. XAP2 delays nuclear translocation of GR. HeLa cells were transfected with 0.25 μg of a plasmid encoding GR-GFP together with 0.75 μg of either vector control or wtXAP2 plasmid. After one day, cells were exposed to 10 nM cortisol, fixed after the indicated times and the nucleus/cytosol distributions were scored for each time point in two independent experiments.

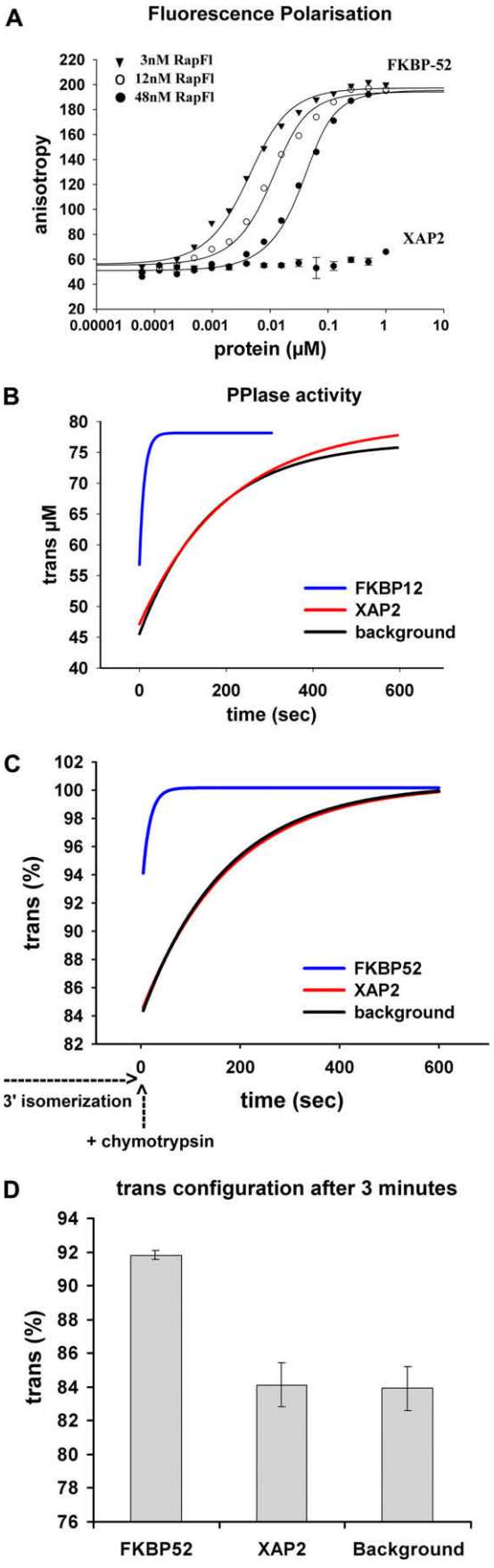
To measure the PPIase activity, we employed a chymotrypsin-coupled enzymatic assay. As shown in Fig. 4B, FKBP12 displayed clear catalytic activity, while the isomerization rates observed for the Xap2 PPIase-like domain did not differ from the uncatalysed thermal isomerization rate of the control. Whether the Xap2 fragment was still fused to MBP, or had been cleaved off by factor Xa, made no detectable difference. To rule out the possibility that MBP or factor Xa have a general inhibitory effect on PPIase activity, we also added FKBP12 as a positive control, but its PPIase activity was not affected.

It was possible that XAP2 shows no activity, because its PPIase domain was degraded by the protease chymotrypsin during the assay. Since we could not exclude this possibility by gel electrophoresis (data not shown), we performed a discontinuous PPIase assay. The FKBP-catalyzed or background cis–trans re-equilibration was induced by a rapid shift of the substrate from dry trifluoroethanol/LiCl to aqueous buffer in the absence of chymotrypsin. The amount of formed trans-substrate was determined after defined time points by the addition of chymotrypsin and fitting of the pNA-generation progress curve (displayed for the additions after 3 min in Fig. 4C). The percentage of trans-substrate increased from an initial 70% trans substrate to the expected equilibrium of 90% trans within 10 min (data not shown). Importantly, at intermediate time points, FKBP52-containing samples clearly equilibrated faster than XAP2-containing samples, which were undistinguishable from background samples (Fig. 4D).

Thus, the actions of XAP2 on GR, as well as the other documented actions of XAP2, are independent of peptidyl prolyl isomerisation.

4. Discussion

The discovery of TPR proteins like FKBP52, FKBP51, CHIP and PP5 as important regulators of GR raises the question of whether other TPR proteins would also have the potential to influence GR. We show here that XAP2 specifically reduces GR's transcriptional



activity in mammalian cells in an Hsp90-dependent manner. Our data suggest that a reduction in ligand-binding affinity, and maybe to some degree also a delay in nuclear accumulation of GR, is involved in the mechanism of this inhibitory effect. XAP2 left the protein levels of GR unchanged.

XAP2 showed no effect on GR in yeast in an earlier study [23]. This is congruent with our discovery in mammalian cells, because in contrast to mammalian cells, GR activity is suboptimal in yeast, possibly due to the lack of some TPR cofactors. As a consequence, FKBP51, which inhibits GR in mammalian cells, has no effect in yeast [7,11].

We show here that, unlike the FKBPs and despite its homology to the large FKBPs, Xap2 does not bind to rapamycin and displays no PPIase activity. Initially, it has been proposed that the PPIase activity is important for the regulatory effect of FKBP52 in GR signalling [7,11]. However, it has been shown more recently that a specific structure of the PPIase domain is the relevant feature, rather than the enzymatic activity itself [40]. In addition, the PPIase activity of FKBP51 is also not required for the inhibitory action of this immunophilin on GR in mammalian cells [7]. Thus, the role, if any, of peptidyl prolyl isomerisation in the regulatory function of the steroid receptors by the FKBPs is not yet clear. As for FKBP51, no specific biochemical function is known for XAP2 that is essential for the inhibitory effect on GR. It is possible that these two factors simply act as competitors that at certain expression levels expel crucial factors from the TPR acceptor site of Hsp90. XAP2 may not be the most prominent regulator of GR, but could become relevant for GR under certain physiological conditions. For example, XAP2 is widely expressed, but varies greatly between tissues, and activation and nuclear translocation of AhR could disengage XAP2 which could then affect GR [41].

Like FKBP51, XAP2 also delays nuclear translocation of GR upon hormone stimulation, but only to a small degree. We measured this effect at sub-saturating levels of cortisol. Since the effect was only small short-term and disappeared after about 1 h, it is less likely to be explained by an effect of XAP2 on hormone binding affinity. Our assay conditions were not sensitive enough to detect an effect, if any, of XAP2 on GR-dependent transcription after very short exposure times (data not shown). Therefore, the physiological relevance of the small delay in nuclear translocation is unclear.

After demonstration of a regulatory role of XAP2 for AhR [24,42] and the hormone receptor TRβ1 [30], our study adds with GR another nuclear receptor affected by XAP2. Whether XAP2 has the ability to act on other members of the nuclear receptor family, remains to be determined.

Fig. 4. The PPIase-like domain of XAP2 does not bind to rapamycin and is enzymatically inactive. (A) The PPIase-like domain of XAP2 (fragment 121) was expressed as a soluble MBP fusion protein in *E. coli* and then affinity purified over an amylose column. Binding of a fluorescent ligand (Rap-Fl) to the target protein was analyzed by a fluorescence polarization assay. FKBP52 was used as a control with different concentrations (3, 12, 48 nM) of Rap-Fl. The experiment with the XAP2-fragment was conducted in duplicate at 48 nM of Rap-Fl. (B) PPIase activity of purified XAP2 (fragments 121) was assessed in a chymotrypsin-coupled enzymatic assay. The release of pNitroanilide from the trans form of the substrate was monitored in a spectrophotometer with time. FKBP12 was added to the protein solution as a positive control. (C) Isomerisation reactions were started in the absence of chymotrypsin, which was added 3 min later to record the % trans conformation. (D) Quantification of the % trans conformation after 3 min in the absence of chymotrypsin. Mean values ± mean errors of 3 independent experiments are displayed.

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